Analysis of *Juniperus phoenicea* from throughout its range in the Mediterranean using DNA sequence data from nrDNA and petN-psbM: The case for the recognition of *J. turbinata* Guss.

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ABSTRACT

DNA sequences were analyzed from 19 populations of *J. phoenicea* from throughout its range. The sequence data (nrDNA, petN-psbM) revealed that *J. phoenicea* is clearly divided into two taxa. These taxa have been recognized as var. (subsp.) *phoenicea* and var. (subsp.) *turbinata* by Adams (2011) and Farjon (2005). However, the magnitude of the differences in the DNA regions, along with the differences in pollen shedding times, morphology and prodelphinidin content support the recognition of *J. turbinata* Guss. No differentiation was found between the typical Mediterranean and Canary Island populations, offering no support for the recognition of *J. phoenicea* subsp. *canariensis* (Guyot) Rivas-Martinez. *Juniperus turbinata* appears to be widespread from Madeira - Canary Islands to the Sinai with few DNA differences among most populations. However, some populations (Grazalema, Madeira, Sinai, central Italy) had moderate amounts of divergence (3-4 mutations) and warrant additional study.
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KEY WORDS: *Juniperus phoenicea* var. *phoenicea,* var. *turbinata, Juniperus turbinata,* phylogeny, *Cupressaceae*, DNA, nrDNA (ITS), petN-psbM, geographic variation.

The genus *Juniperus* is comprised of approx. 75 species in 3 sections (Adams, 2011) with serrate (denticulate) leaf-margined species found in both the eastern hemisphere (1 species) and western hemisphere (21 species). *Juniperus phoenicea* is the only serrate-leaf juniper in the eastern hemisphere and generally treated as *J. p.* var. *phoenicea* and var. *turbinata* (Adams, 2011) or as subsp. (Farjon, 2005). However, Adams and Schwarzbach (2013) have recently shown that *J. phoenicea* is not part of a clade of serrate-leaf junipers occurring in the western hemisphere, leading them to denote *J. phoenicea* as a 'pseudoserrate' juniper. In addition, they found *J. p.* var. *phoenicea* and var. *turbinata* to be as different in their DNA sequences as several other recognized species of *Juniperus*; lending support for the recognition of *J. turbinata* Guss. as proposed by Lebreton and Perez de Paz (2001) based largely on the concentration of prodelphinidin, a polymeric tannin. The prodelphinidin data suggested that *J. phoenicea* var. *phoenicea* was confined to the Iberian Peninsula (Fig. 1), with var. *turbinata* being widespread throughout the Mediterranean (Fig. 1). However, Farjon (2005) considered subsp. *phoenicea* to be widespread in the Mediterranean and subsp. *turbinata* to be confined to littoral maritime habitats (sand and rocks). Adams (2011) followed the distributions of Farjon (2005), except for the Canary Islands and Madeira, which, based on DNA sequence data, have been shown to be var. *turbinata* (Adams et al. 2010).

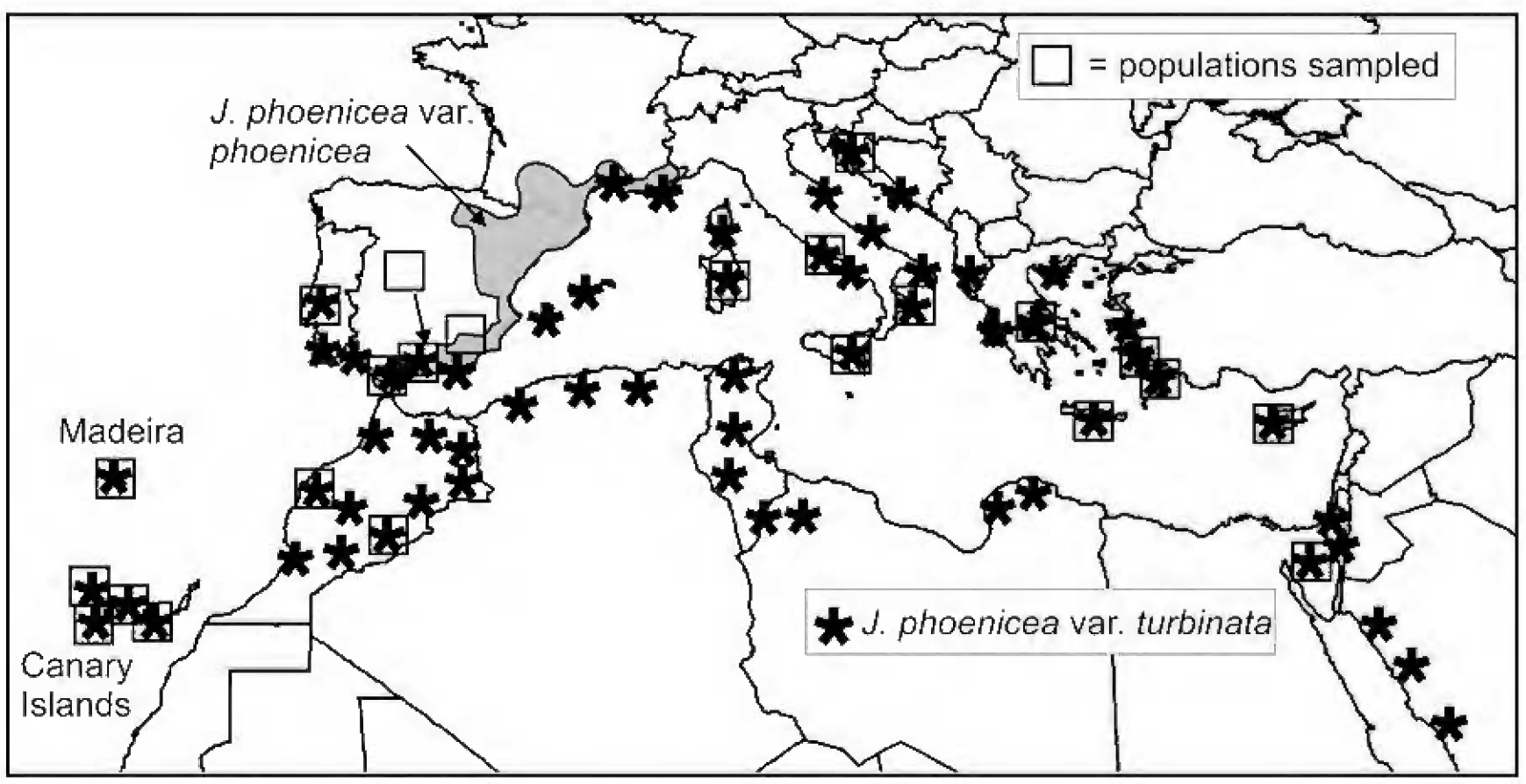

Figure 1. Distribution of *J. phoenicea* (adapted from Lebreton and Perez de Paz (2001) and Adams et al. (2010). Squares show populations of vars. *phoenicea* and *turbinata* sampled in the present study.

Arista and Ortiz (1995) analyzed plants from Sierra de Grazalema, Spain and reported the seed cones of subsp. *turbinata* were larger (7.5 mm L x 8.8 mm W) than subsp. *phoenicea* (6 mm L x 5.8 mm W) at that location. Arista, Ortiz and Talavera (1997) analyzed the reproductive isolation of subsp. *phoenicea* and *turbinata* in the Sierra de Grazalema populations. They reported flowering (pollen shedding) occurred in the fall (Oct.-Nov.) for subsp. *turbinata* and in the spring (Feb.-March) for subsp. *phoenicea*, effectively preventing cross-pollination in these (normally) monecious taxa. Interestingly, subsp. *phoenicea* grew on dolomitic soil whereas subsp. *turbinata* was found on Cambrian limestone (in contrast to coastal sand dunes in southern Spain).

Mazur et al. (2003, 2010) compared several populations of var. *turbinata* (Portugal, sw Spain, Italy and Morocco) and of var. *phoenicea* from ne Spain using 14 morphological characters. They did not find the seed cone length or width to be very different, but the ratio of cone length/width (i.e., shape) seemed to discriminate between the taxa. Perhaps the best character (Mazur et al. 2003) was the number of seeds/ cone (4.97 - 5.96 for var. *turbinata* vs. 8.05 for var. *phoenicea*).

Table 1. Morphological differences between var. *phoenicea* and var. *turbinata*.

	var. *phoenicea*	var. *turbinata*
seed cones	spherical to globular	elongate or turbinate (when immature) almost spherical (when mature) in some populations.
	smaller, 5-9 mm long	larger, 7-11mm long
number of seeds	more, 3(7-9)13	fewer, 3(4-7)10
pollen shed	spring (Feb. - March)	fall (Oct.-Nov.)
branchlets	thicker	thinner
branchlets bark	gray to brown	reddish
habitat	dolomitic soil	sand, Cambrian limestone, volcanic rock

Adams et al. (2002) utilized RAPDs to compare *Juniperus phoenicea*, *J. p.* var. *canariensis*, *J. p.* subsp. *eu-mediterranea*, and *J. p.* var. *turbinata* from El Peñón, Spain, Setubal, Portugal, Corse (from high and low α-pinene plants), the Canary Islands (Tenerife), Nea Epidavios, and Delphi, Greece, and the Tarifa sand dunes, Spain. They found the high and low α-pinene plants from Corse clustered together, along with other var. *turbinata* populations. The var. *phoenicea* plants from El Peñón, Spain formed a separate cluster, with all the other populations clustering with var. *turbinata* from the Tarifa sand dunes, Spain (Adams et al., 2002). They concluded that *J. p.* var. *canariensis* and *J. p.* subsp. *eu-mediterranea* were not distinct taxa but included in *J. p.* var. *turbinata.*

A second study with RAPDs data (Adams et al., 2006) compared *J. phoenicea* from the Canary Islands with plants of var. *turbinata* from Morocco and the Tarifa sand dunes, Spain plus var. *phoenicea* from El Peñón, Spain. They found the plants from the Canary Islands, Morocco, and Tarifa sand dunes clustered together, whereas var. *phoenicea* from El Peñón, Spain formed a separate cluster.

Dzialuk et al. (2011) also used RAPDs to compare plants from Andorra, France, Morocco, Portugal and 3 sites in Spain. Principal coordinates clearly separated 3 of the subsp. *phoenicea* populations from subsp. *turbinata* (Fig. 2, Dzialuk et al., 2011), but one population of putative subsp. *phoenicea* from Spain (SP 3) was ordinated with populations of subsp. *turbinata.* Interestingly, previous work (Boratynski et al., 2009), using isozyme data, found SP 3 to cluster with other populations of subsp. *phoenicea* from Spain. In fact, Boratynski et al. (2009, Fig. 3) showed all populations of subsp. *phoenicea* from Spain and France clustered together and populations of subsp. *turbinata* from Greece, Italy, Morocco, Portugal, Spain and Turkey formed a separate cluster.

The purpose of the present study was to examine DNA sequence data from nrDNA and petN-psbM regions for individuals of *J. phoenicea* from throughout its range, to determine if the two taxa are distinct and if they are distributed as suggested by the prodelphinidin data of Lebreton and Perez de Paz (2001), see Fig. 1 above.

MATERIALS AND METHODS

Specimens used in this study: **var. *phoenicea*:**
Spain, El Peñón, 37 ° 35' 38" N, 3 ° 31' 22" W, elev. 760m, *Adams 7077-7079*,
Spain, Sierra de Grazalema, 36° 47' 51.5" N, 5°24' 43.7"W, 835 m; *M. Arista 1-5,* Baylor specs. *Adams 13813-13817.*
var. *turbinata*:
Canary Islands, Tenerife, 0.5km S.of Tejina de Isora on rt.822, 29° 10' 48"N, 16° 45' 53"W, ca. elev. 520m, *Adams 8147-8149*

Corse, France, *Joe Casanova 1-3*, *Adams 8893-8895,*
Crete, Dragonada Isl., 35° 22' 32" N; 26° 11' 01" E. elev. ca. 30 m, *Avramakis Manolis 1-2,* Baylor specs. *Adams* 13605-13606,
Croatia, Ugljan Island, 44° 05' 0.27" N, 15° 09' 39.29"E, elev. 20-32 m, *Zlato Liber 1-5*, Baylor specs. *Adams 13589-13593,*
Cyprus, CYP-1 35° 00' N, 32° 18' E elev. 400 m, *Adam Boratynski CYP-1(1-5),* Baylor specs. *Adams* 13351-13355,
Cyprus, CYP-2 34° 58' N, 34° 04' E, elev. 20 m, *Adam Boratynski IT-1(1-5),* Baylor specs. *Adams 13356-13360*
Italy, central, Sabaudia, 41 ° 15' N, 13 ° 02" E, elev. 10 m, *Adam Boratynski IT-1(1-5),* Baylor specs. *Adams 13336-13340,*
Italy, southern, Crotone, 38° 53' 36" N, 17° 05' 42" E, elev. 10 m, *Adam Boratynski IT-2(1-5),* Baylor specs. *Adams 13341-13345,*
Madeira Island, Portugal, elev. ca. 20m, *Adams 11502-11504,*
Morocco, rd to Oukaimeden, 31° 21.033'N, 07° 45.893'W, elev. 940m, *Adams 9408-9410*
Morocco, Essaouria sand dunes,31° 29' 26"N, 9° 44' 29" W, elev. 98m, *Adams 10407-10408*,(ex Nadia Achak),
Portugal, Setubal, *Adams 7074-7076,*
Sicily, near Piano Pirrera near Acate (Ragusa), 37° 01' 35.75" N; 14° 26' 07.86" E., 120 m, *Pietro Minissale & Saverio Sciandrello 1-5,* Baylor specs. *Adams 13778-13782*
Sinai, 30°38'09"N, 33°26'53"E, elev. 700 m *Hagar Leschner 1-5,* Baylor specs. *Adams 13495-13499,*
Spain, Sierra de Grazalema, 36° 48' 10.9"N, 5° 24' 21.2"W, elev. 829m, *M. Arista 6-10,* Baylor specs. *Adams* 13818-13822,
Spain, Tarifa sand dunes, elev. ca. 20m, *Adams 7202-7204,*
Turkey, Orak Island, Bodrum-Mugla Province, 36° 58' 25"N, 27° 35' 45" E, elev. 44 m, *Tugrul Mataraci T-1*, Baylor specs. *Adams 12397,*
Turkey, Marmaris Peninsula, 36° 49' N, 27° 50' E, elev. 700 m, *Adam Boratynski Tu-1(1-5),* Baylor specs. *Adams 13346- 13350,*
***Juniperus sabina* (outgroup):** Switzerland, Baltschieder, 1300m, *Adams 7611-7612,*
Voucher specimens are deposited at BAYLU herbarium Baylor University.

One gram (fresh weight) of the foliage was placed in 20 g of activated silica gel and transported to the lab, thence stored at -20° C until the DNA was extracted. DNA was extracted from juniper leaves by use of a Qiagen mini-plant kit (Qiagen, Valencia, CA) as per manufacturer's instructions.

Amplifications were performed in 30 µl reactions using 6 ng of genomic DNA, 1.5 units Epi-Centre Fail-Safe Taq polymerase, 15 µl 2x buffer E (petN, trnD-T, trnL-F, trnS-G) or K (nrDNA) (final concentration: 50 mM KCl, 50 mM Tris-HCl (pH 8.3), 200 µM each dNTP, plus Epi-Centre proprietary enhancers with 1.5 - 3.5 mM $MgCl_2$ according to the buffer used) 1.8 µM each primer. See Adams, Bartel and Price (2009) for the ITS and petN-psbM primers utilized. The primers for trnD-trnT, trnL-trnF and trnS-trnG regions have been previously reported (Adams and Kauffmann, 2010).

The PCR reaction was subjected to purification by agarose gel electrophoresis. In each case, the band was excised and purified using a Qiagen QIAquick gel extraction kit (Qiagen, Valencia, CA). The gel purified DNA band with the appropriate sequencing primer was sent to McLab Inc. (San Francisco) for sequencing. Sequences for both strands were edited and a consensus sequence was produced using Chromas, version 2.31 (Technelysium Pty Ltd.) or Sequencher v. 5 (genecodes.com). Sequence datasets were analyzed using Geneious v. R6-1 (Biomatters. Available from http://www.geneious.com/) and the MAFFT alignment program. Further analyses utilized the Bayesian analysis software Mr. Bayes v.3.1 (Ronquist and Huelsenbeck 2003). For phylogenetic analyses, appropriate nucleotide substitution models were selected using Modeltest v3.7 (Posada and Crandall 1998) and Akaike's information criterion.

Minimum spanning networks were constructed from mutational events (ME) data using PCODNA software (Adams et al., 2009; Adams, 1975; Veldman, 1967).

RESULTS AND DISCUSSION

Sequencing nrDNA (nuclear ribosomal DNA) ITS regions yielded 1276 bp of data. Sequencing petN-psbM (intergenic region of chloroplast DNA) provided 855 bp of data. Combined, these data afforded 2131 bp of data. A Bayesian tree (with *J. sabina* as an outgroup) shows (Fig. 2) strong support for var. *phoenicea* and var. *turbinata* as previously reported (Adams and Schwarzbach, 2013). Most of the var. *turbinata* populations displayed very little variation. Exceptions were: one plant from Delphi, Greece which is in a distinct clade with Cyprus plants; whereas the other Delphi plant is in another clade; and the plants from Sinai, Madeira and Grazalema are separate from the major clade of var. *turbinata* (Fig. 2).

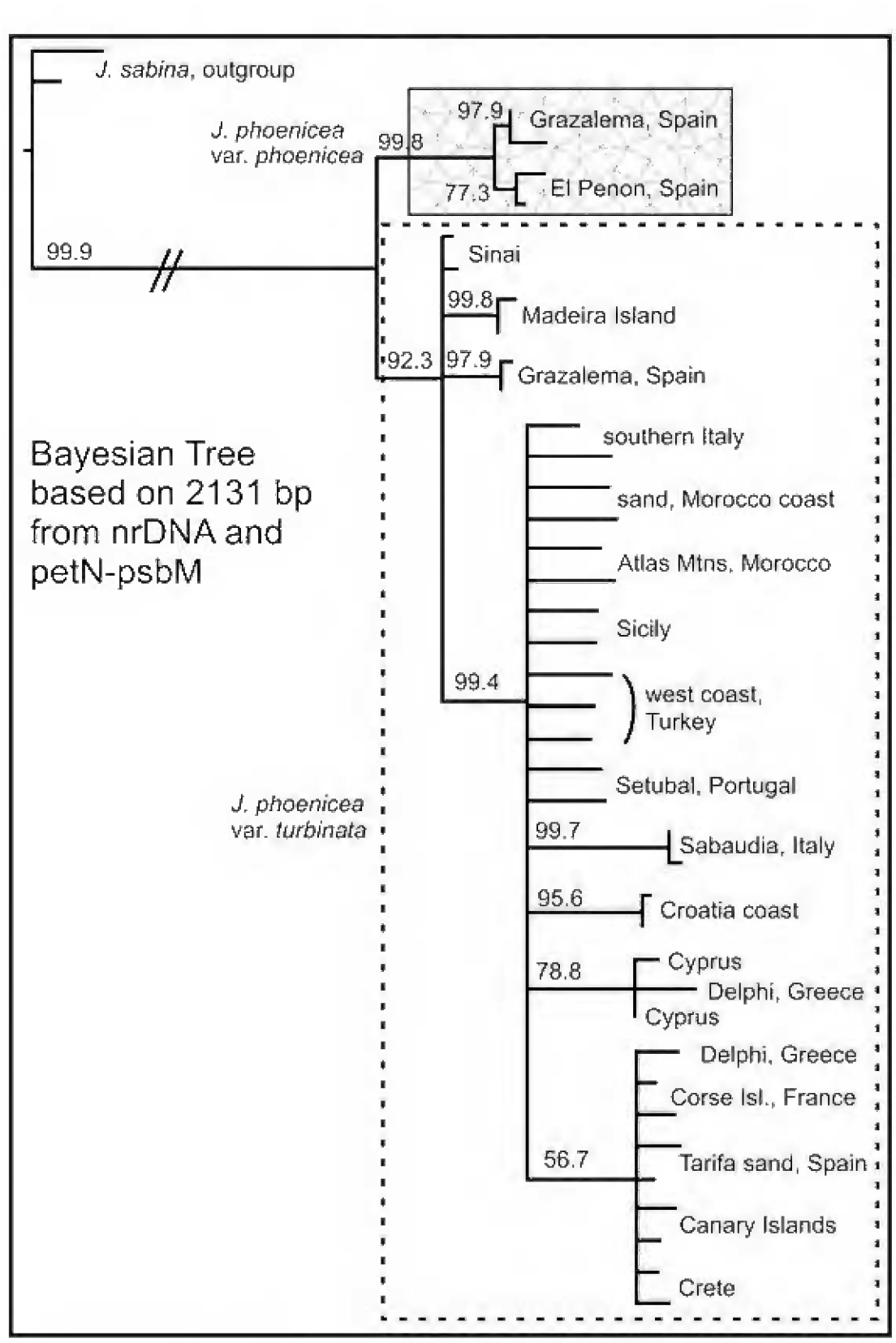

Figure 2. Bayesian tree based on nrDNA and petN-psbM sequences. The numbers at the branches are posterior probabilities (as percents).

To examine the magnitude of the differences among the populations, a minimum spanning network was constructed (Fig. 3). The outgroup (*J. sabina*) is quite distant (65 MEs, Fig. 3) as previously shown by Adams and Schwarzbach (2013). In fact, *J. phoenicea* is not close to any juniper species and certainly not related (unless very distantly) to the serrate-leaf junipers of North America.

Notice that the minimum spanning link between var. *phoenicea* and var. *turbinata* is 13 MEs, based on data from only 2 DNA sequences. This is a very large difference, comparable to species differences in section *Sabina* (Adams and Schwarzbach, 2012). Many of the populations of var. *phoenicea* differ by only 0 or 1 MEs (Fig. 3). A few of the populations differ from the central group by 4 MEs: Grazalema, Spain; central Italy; Madeira Island and Sinai.

There is some variation in var. *phoenicea* with plants at El Peñón differing by 2 MEs and the Grazalema plants are separated by 3 MEs from nearby El Peñón (Fig. 3).

Plotting the minimum spanning network onto a geographic map offers additional perspective (Fig. 4). The major feature of the network is the nearly identical DNA sequences (i.e., 0, 1 or 2 MEs) between most of the populations. The Sicily population, particularly representative as it is probably the largest on the island (Minissale and Sciandrello 2013), appears to be the most central of the nodes with no (0) MEs to Morocco and southern Italy, and by only 1 ME to Tarifa, Spain and Turkey populations. In addition, the Canary Islands population had no differences from the Tarifa, Spain population (Fig. 4).

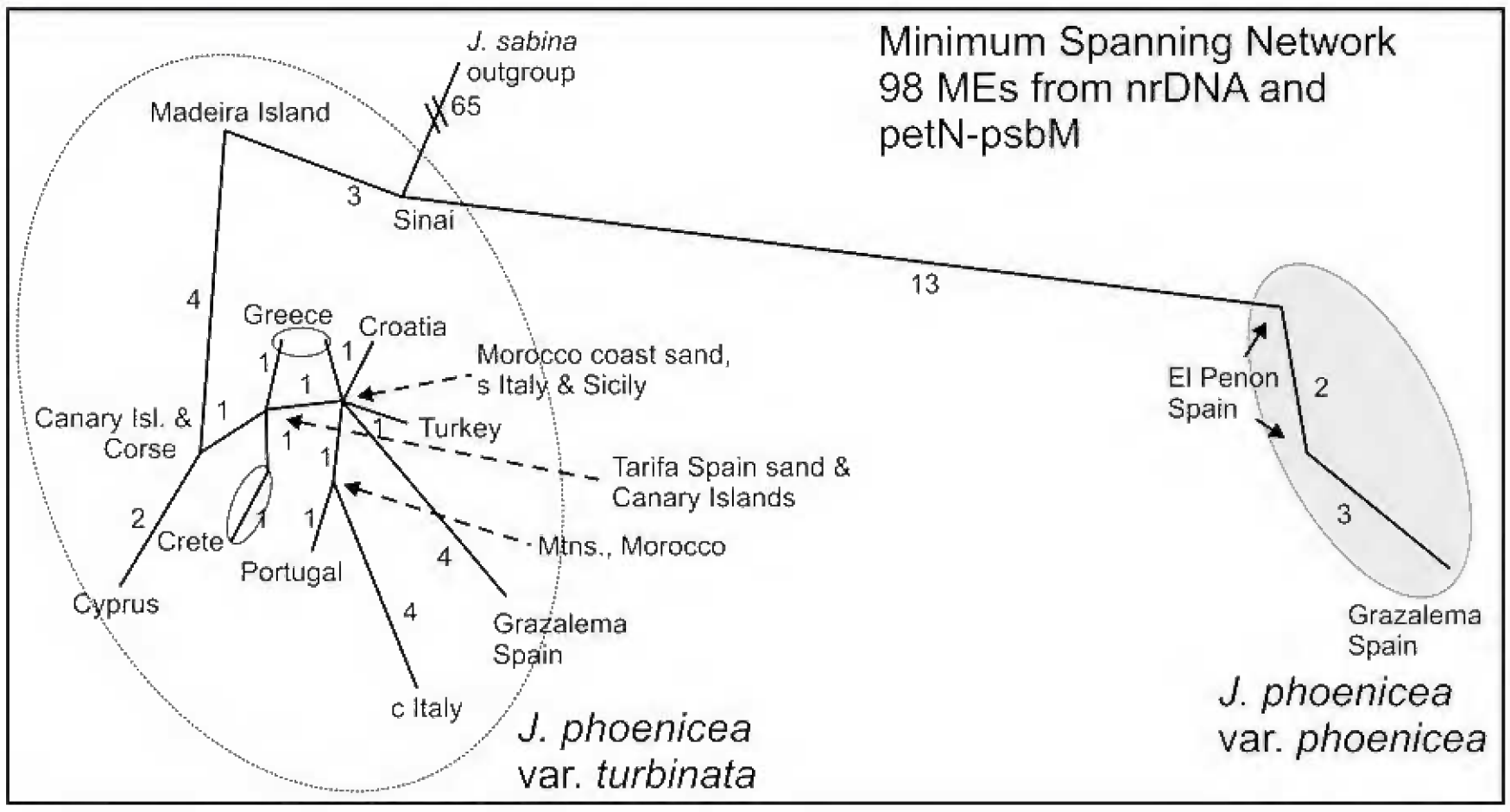

Figure 3. Minimum spanning network based on nucleotide substitutions and indels (Mutational Events, MEs). Numbers next to links are the number of MEs.

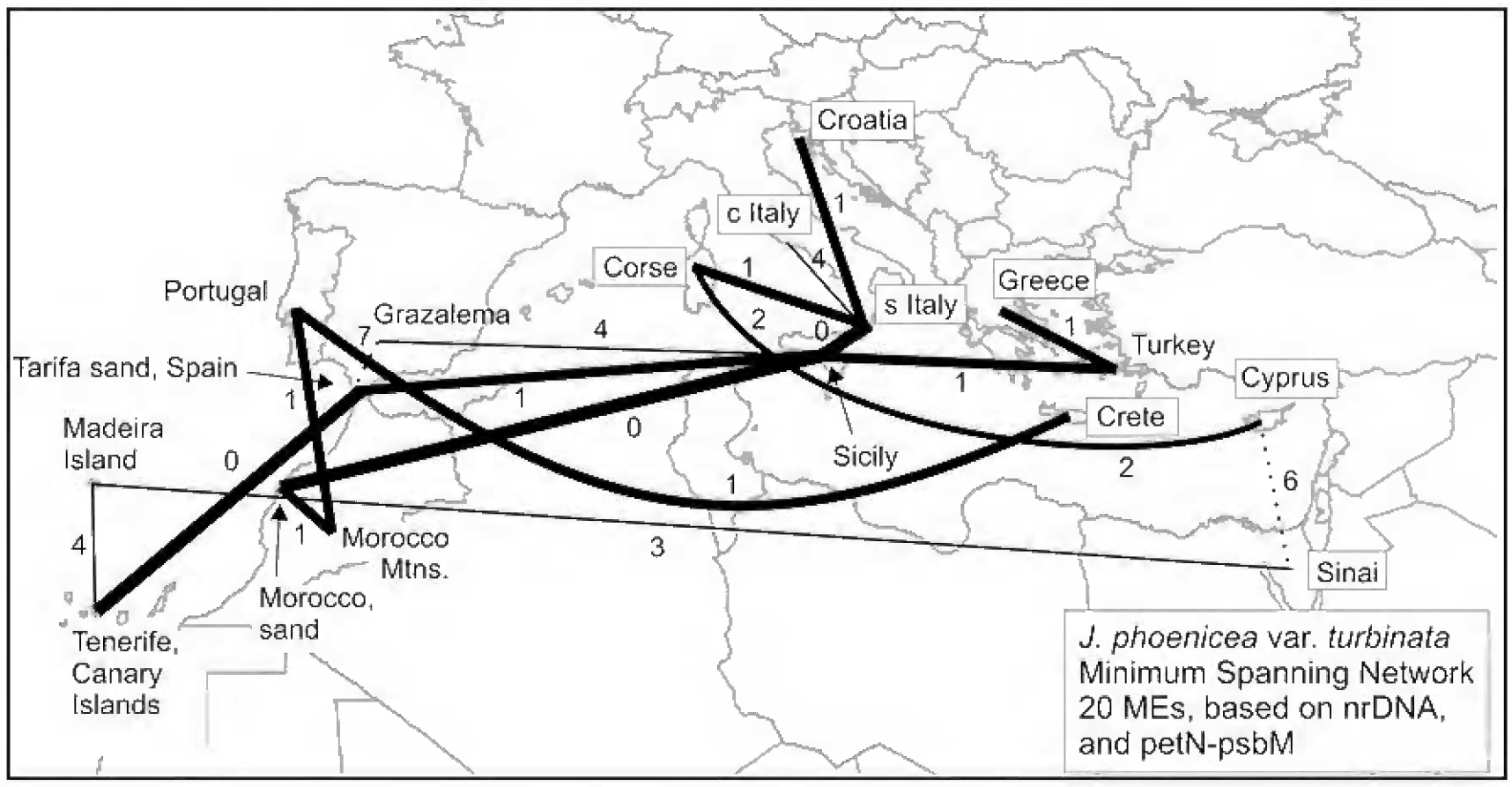

Figure 4. Minimum spanning network plotted onto a geographic map. Numbers next to lines are the number of MEs for the link. The width of a line is proportional the similarity between nodes. The widest lines denote no differences (0 MEs), whereas the narrowest lines show the least similar nodes (3 or 4 MEs). Dotted lines are links to the nearest geographic population.

Several populations have 3 or 4 ME differences from their nearest node. Grazalema, Spain differs by 4 MEs from Sicily (its nearest or most similar neighbor) and by 7 MEs to the nearby Tarifa sand population (Fig. 4). Because both var. *phoenicea* and var. *turbinata* co-occur at the Grazalema site, one might suspect that hybridization may be the cause of the unusual differentiation in var. *turbinata.* But, because they shed pollen in the spring and fall, respectively, that should not be a factor. The nrDNA (ITS) sequences for var. *phoenicea* and var. *turbinata* from Grazalema differ by 6 nucleotide substitutions and 4 indels. If hybrids are involved, one would expect to find some of the substitution differences to be polymorphic. However, re-examination of the sequencing chromatograms of these plants failed to reveal heterozygous peaks, implying that hybridization is not involved in the divergence of the var. *turbinata* at Grazalema from other populations.

Madeira Island plants differ by 4 MEs from the Canary Islands plants and 3 MEs from the Sinai plants (Fig. 4). The DNA differences for Madeira - Canary Islands parallel the differences found in *Juniperus cedrus* from Madeira and Canary Islands (9 MEs, nrDNA + petN-psbM, Fig. 7, Adams et al., 2010). Combined with leaf terpenoid differences, Adams et al. (2010) recognized *J. maderensis* on Madeira. However, the present differences (4 MEs in nrDNA + petN-psbM) are not as great, so it is premature to recognize the Madeira var. *turbinata* as a different variety. The divergence of the small population on Madeira may be the results of a founder event or genetic drift. Additional research on leaf terpenoids (in progress) may help resolve this taxonomic question.

It is interesting that the Sinai population is most closely linked to Madeira (3 MEs, Fig. 4), but it is 6 MEs distant from the nearby Cyprus population. It seems improbable that seeds were transported between the Sinai and Madeira populations. Perhaps research on leaf terpenoids (in progress) will help illuminate this problem.

Finally, the central Italy (Sabaudia) population differs by 4 MEs from the southern Italy plants. Boratynski et al. (2009) included the central Italy population (IT-1) in their study using isozymes. They found it to cluster closely with Greece (GR-1) and Morocco (MOR). The central Italy population is on old coastal sand dunes. The population is large and not too distant from other populations in Italy, Sicily, Corse and Croatia (Figs. 1, 4). Perhaps variation of 3-4 MEs might be expected. Additional research is needed.

CONCLUSION

In summary, this study indicates that *J. phoenicea* is clearly divided into two taxa. These taxa have been recognized as var. (subsp.) *phoenicea* and var. (subsp.) *turbinata* by Adams (2011) and Farjon (2005). However, the magnitude of the differences in the DNA regions sequenced in this and Adams and Schwarzbach (2012), along with the differences in pollen shedding times, morphology and prodelphinidin (Lebreton and Perez de Paz, 2001) support the recognition of *J. turbinata* Guss. No differentiation was found between the typical Mediterranean and Canary Island populations, offering no support for the recognition of *J. phoenicea* subsp. *canariensis* (Guyot) Rivas-Martinez.

Juniperus turbinata appears to be widespread from Madeira - Canary Islands to the Sinai with few DNA differences among most populations. However, some populations (Grazalema, Madeira, Sinai, central Italy) show moderate amounts of divergence (3-4 mutations) and deserve additional study.

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